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Fitting abnormal oxygen equilibrium curves of hemoglobin

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A growing number of oxygen equilibrium curves for hemoglobin (Hb) mutants, post-translational modifications, or the binding of potent new effectors of Hb cannot be fitted adequately with the two-state model. Examples are curves showing double maxima in the derivative of the Hill plot, or slopes of less than unity. We present such examples of modified hemoglobins and strong effectors in this study and calculate at which substate level the two-state model differs from the data. Analysis of hemoglobin oxygen equilibrium curves is reconsidered using the two-state model extended to allow variation of the individual substate probabilities. In this way the effect on the equilibrium due to perturbations in energy of each substate can be studied as a diagnostic tool.

1. Introduction

The two-state model [1] provides a useful description of hemoglobin (Hb) oxygenation as starting in a low-affinity (T) state with no ligands bound and undergoing an allosteric transition in a well-defined manner towards a high-affinity (R) state. Since only two parameters determine the shape of the oxygen equilibrium curve (OEC), the model is limited in the variety of OEC it can generate [2]. Generally, the two-state model provides good simulations of ligand binding to Hb and offers a physical model for discussion of the different environments (pH, effectors) and Hb mutants [3].

One basic question is how to proceed when the two-state model fails to simulate the data within the required precision. This has been a difficult problem, as the alternative to the simple model often involves a large number of substates. A two-state model implies 10 substates: two allosteric forms, each capable of binding from zero to four ligands. The equilibrium between the states is rigidly defined, resulting in only three parameters to describe the ligand binding. Since this model already provides a good approximation, it is clearly difficult to provide precise determinations of all variables at the 10-state level. Thus, most work has considered different relations between the states, such as an additional heme-heme interaction [4,5] or additional allosteric states in the case of effectors [6–8]. Any new allosteric state or interaction energy results in new substates.

The foundation for a detailed analysis of allosteric systems at the substate level has been established by Wyman [9]. In this study, we consider the effect of perturbing the energy of one of the original 10 substates. This permits the use of the simple two-state framework and provides a much larger variation of the possible equilibrium curves which can be simulated.

This paper is dedicated to J. Wyman in honor of his pioneering contribution to the physical chemistry of allostery in proteins and particularly in hemoglobin.

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2. Methods

2.1. Experimental

Oxygen equilibrium curves (OEC) for purified human Hb A were measured using a continuous method as previously described [7]. Standard experimental conditions were 0.1 M NaCl, 50 mM Bis-Tris buffer (pH 7.2) at 25°C. The experimental data were then analysed and fitted to the two-state formulation using an iterative least-squares procedure.

Glycerated Hb, β-82(EF6)-*N*-glyceryllysine, was prepared from the blood of a patient deficient in 2,3-diphosphoglycerate mutase [10]; samples showed an increased p_{50} and a lower Bohr effect relative to Hb A. Less than 3% met-Hb was present in the purified samples.

OEC were also recorded for the reaction of Hb A with the new potent effector L345 (2-[4-(3,4,5-trichlorophenylureido)phenoxy]-2-methylpropionic acid) [11–13]. OEC were measured at effector concentrations of 0.05–2 mM.

2.2. Simulations

Within the two-state framework, the substate energy levels for a particular allosteric form are equally spaced; i.e., each additional ligand shifts the allosteric equilibrium by the same factor:

$$L = T_0/R_0;\ T_i/R_i = Lc^i \qquad (1)$$

where L is the equilibrium coefficient for the deoxy tetramer and the equilibrium shifts by a factor $c = K_R/K_T$ for each ligand bound. i denotes the number of ligand molecules bound. This three-parameter representation of the data describes differences in OEC as shifts in the equilibrium between the two quaternary forms or as changes in the affinity of the pure states [8].

For a given set of parameters, the relative probabilities for the 10 tetramer substates can be calculated:

$$R_i = s_i\alpha^i R_0 \quad T_i = s_i\alpha^i L c^i R_0 \qquad (2)$$

where the statistical factor $s_i = 4!/[(4-i)!i!]$ and $\alpha = [\text{ligand}]/K_R$. What happens if one substate is slightly displaced from the two-state scheme? We consider such displacements or perturbations by using a computer program which allows variation of individual substate energies.

The method consists firstly of the determination of the relative probabilities for all substates within the two-state framework (R_i and T_i). A given substate probability can then be modified by a factor p; all other substates keep their relative contribution to the total normalized amount of Hb.

$$Y = (1/4)(1/N)\sum_{i=1}^{4} i(p_{T_i}T_i + p_{R_i}R_i) \qquad (3)$$

where the normalisation factor $N = \sum_{i=0}^{4}(p_{T_i}T_i + p_{R_i}R_i)$. A given substate can be perturbed to give it a slightly higher or lower probability relative to the two-state populations in order to determine its contribution to the shape of the OEC. The stepwise Adair scheme can also provide differences at each ligation level, but does not consider the R and T substates of the two-state model. This type of analysis or diagnostic tool allows the determination of how the overall OEC depends on each substate and whether the observed data are sensitive to small displacements of the energy level of a given substate.

3. Results

3.1. Simulations

We first consider a variation of the relative probability of the substate T_2, i.e., doubly liganded T-state tetramer. Simulated OEC are shown in fig. 1, for the increase in probability by the factor indicated. The maximum amount of T_2 occurs at 50% ligand saturation ($Y = 0.5$). The four curves shown in fig. 1 have a maximum amount of T_2 of 5, 40, 60, and 85%; i.e., for the highest increase in probability of T_2, the sample would contain 85% of the Hb tetramers in the T_2 form at 50% ligand saturation. As the probability of T_2 is increased, the second ligand is bound at a lower oxygen partial pressure, while the third ligand requires a higher pressure than for the unperturbed case.

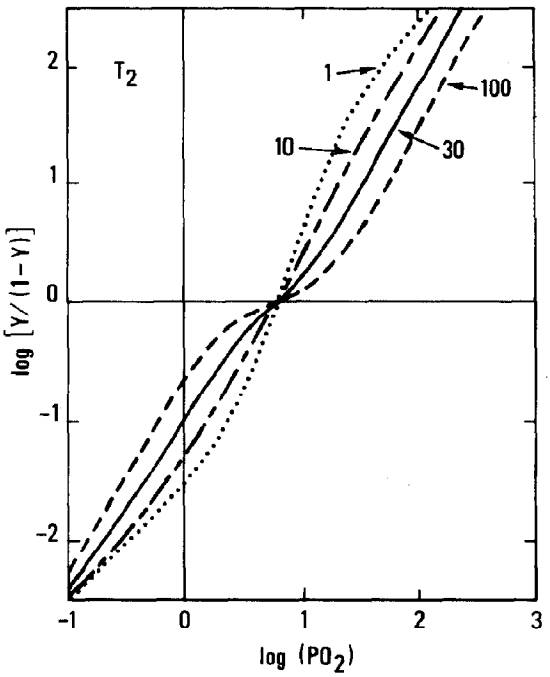

Fig. 1. Hill plot of simulated oxygen equilibrium curves generated from the two-state model ($L = 10^5$, $c = 0.01$, $K_R = 0.3$ mmHg) by increasing the probability for the substate T_2 by the factor shown. The oxygen level for 50% saturation remains fixed.

This reflects the gain in probability of T_2 and the loss in those of all other substates. The increase in probability of T_2 thus results in a lower slope of the Hill plot at 50% saturation. Note that the OEC at 50% saturation is a fixed point independent of variation of doubly liganded forms.

For higher probabilities of T_2, there will necessarily be less R-state tetramer (sum of all R-state forms). This change is shown in fig. 2, along with the fraction of T_2 and the slope of the Hill plot (n). The slope shows a double maximum for large increases in the T_2 probability; the relative minimum between the two maxima is necessarily at 50% saturation.

For small changes in the binding curves, it is more instructive to plot the differences (residuals) in Y. The residuals for various substates are shown in fig. 3. Values shown are the factor increase in probability. A value of 0 indicates that the probability is decreased by at least a factor of 1000 which eliminates the contribution of that state; this cannot be done for the initial and final states T_0 and R_4. All other states can be eliminated to observe their contribution to the OEC, since alternative binding pathways exist. Fixed points in the Hill plot occur at a saturation of $i/4$ for variations of a state with i ligands bound; for example, variation of the substate T_2 does not change the oxygen level required for 50% saturation (see fig. 1).

3.2. Experimental results

The initial analysis is to fit the observed OEC with the two-state model. If the shape of the observed residuals (not necessarily to the best two-state fit) matches one of the forms shown in fig. 3, then a perturbation of one of the 10 sub-

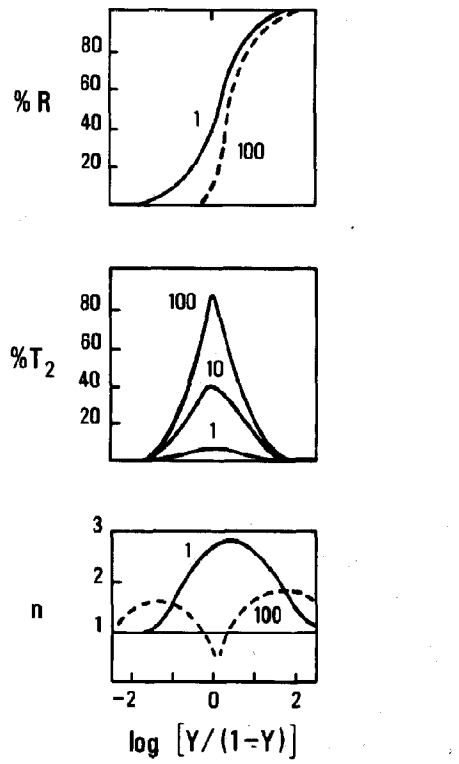

Fig. 2. Effect of the increased probability of substate T_2 on the overall fraction of tetramers in the allosteric state R (upper panel), the fraction of tetramers existing as T_2 (center) and the derivative (n) of the Hill plot (lower).

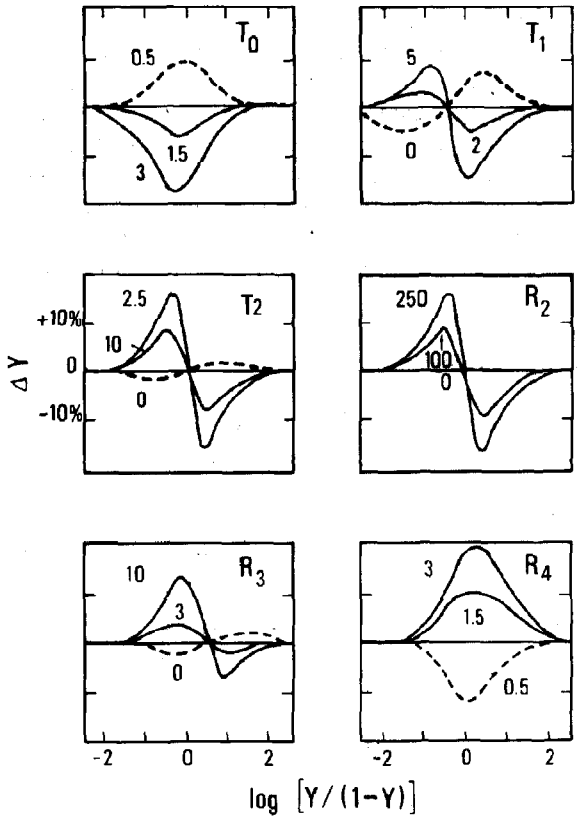

Fig. 3. Residuals in Y for the change in probability of one of the tetramer substates. Values shown are the factor increase in the substate probability relative to the two-state model; a value of zero indicates a large decrease to eliminate the contribution of that state. For this case ($L = 10^5$, $c = 0.01$, $K_R = 0.3$ mmHg), the allosteric equilibrium favors T_2 over R_2 by a factor of 10; thus increases in probability of R_2 by nearly 10-times those of T_2 are needed to produce the same observed difference in the OEC.

states can correct the simulation. The known differences (fig. 3) thus serve as a set of reference residual curves.

3.3. Perturbation of singly liganded tetramers

Glycerated Hb [11] shows an abnormal lower asymptote in the OEC (fig. 4). OEC for glycerated Hb show a higher p_{50} relative to Hb A, and yet a higher observed affinity for the first ligand. This results in a residual with respect to the two-state model similar to that for shifts in the substate T_1

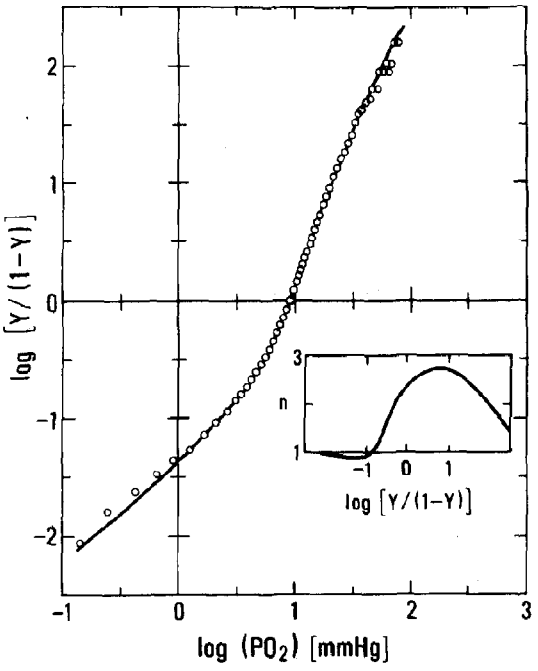

Fig. 4. OEC for glycerated Hb (○) (50 mM Bis-tris buffer at pH 7.2, 0.1 M NaCl, 25 °C) and a simulation using an increase in substate T_1 by a factor of 1.8 ($L = 1.6 \times 10^6$, $c = 0.007$, and $K_R = 0.23$ mmHg). The derivative of the Hill plot (n, inset) shows a value of less than unity near 25% saturation.

(fig. 5). In this case, an increase in the population of T_1 tetramers by a factor of 1.8 could simulate the correct shape of the OEC.

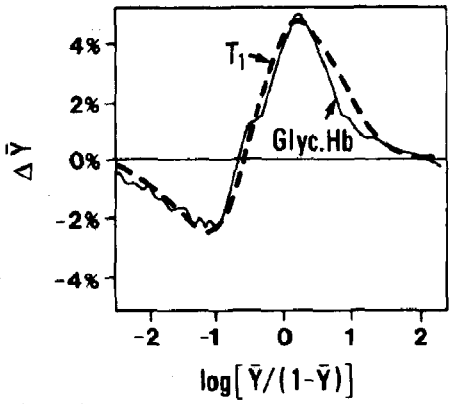

Fig. 5. Residuals in Y for the OEC of glycerated Hb and a two-state simulation (- - - - - -) compared to the difference generated by a shift in probability by a factor of 1.8 for the substate T_1.

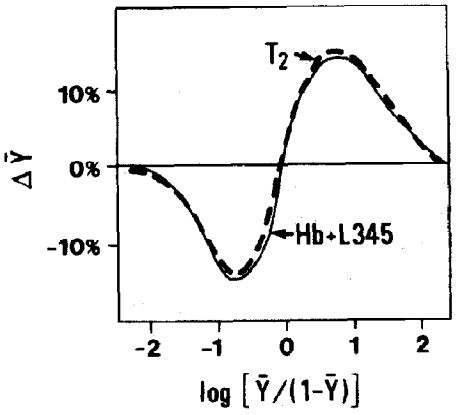

Fig. 6. Residual for the data of Hb + L345 relative to a two-state simulation (- - - - -) and the theoretical residual for a shift by a factor of 17 in T_2.

An additional problem in the analysis of mutant hemoglobins is the presence of unknown amounts of nonallosteric hemes. This could be due to an excess of one type of chain or simply an inactive tetramer. Since these hemes will bind their ligands independently, usually with the high affinity of the R state, the contribution is most evident at low oxygen concentrations where the T-state tetramers

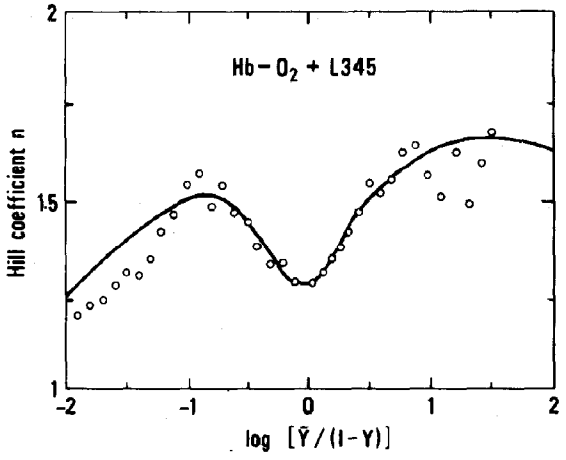

Fig. 7. The derivative of the Hill plot for Hb in the presence of 0.2 mM effector L345 at pH 7 and 25 °C. The curve shows a double maximum in the slope of the Hill plot which can be simulated by an increased probability of the state T_2 by a factor of 17 ($L = 10^6$, $K_R = 1.5$ mm Hg, $c = 0.0063$). The position of the minimum (near $Y = 0.5$) is independent of effector concentration between 0.05 and 1 mM.

are much less saturated. Thus, any trace amounts of such nonallosteric forms tend to mask the lower asymptote for the tetramers.

3.4. Doubly liganded forms

An example of a residual characteristic of a shift in the substate T_2 is shown in fig. 6; the slope of the Hill plot shows a double maximum (fig. 7). The data are for Hb in the presence of the effector L345 [13]. The double maximum in n has been previously observed for nonsaturating amounts of the effectors diphosphoglycerate (DPG) [7,14] and inositol hexaphosphate (IHP) [8]; for these effectors, the position of the relative minimum in n is correlated with the fraction of effector saturation, being at 50% oxygen saturation only at 50% effector saturation. For the case of L345, the shape of the OEC and the position of the relative minimum of n near 50% oxygen saturation are invariant over a wide range of effector concentration (0.05–1 mM), corresponding to a ratio of effector to Hb tetramer of 2 to 40.

4. Discussion

The perturbed two-state model allows one to consider how each substate can affect the overall oxygenation curve. It therefore permits a fine tuning of the simulation if the residuals for simulations using the unperturbed two-state simulation match one of the forms shown in fig. 3. One additional parameter is needed for each probability which is modified; however, it is clear that changing two or more substate energies represents a great departure from the original two-state model.

4.1. Perturbed T_1 substate

Glycerated Hb shows an abnormal lower asymptote; oxygenation curves intersect those of Hb A near 25% saturation, resulting in a residual characteristic of a change in the amount of singly liganded species (fig. 5). Increasing the probability of the substate T_1 by a factor of 1.8 reproduced

the observed shape of the OEC which shows a slope below unity near 25% saturation (fig. 4).

4.2. Double hump effect

The doubly liganded substates are clearly critical in the oxygenation process, as the transition from T to R state occurs under most conditions after two ligands are bound. As shown in fig. 1, changes in the relative probability of the substate T_2 can produce large changes in the shape of the OEC. For sufficiently large increases in the probability of T_2, the derivative of the Hill plot shows a minimum at 50% saturation.

The double maxima can be generated by an increase in the probability for the substate T_2; in this case, the first and last ligand bind with the original T- or R-state affinity, yet the observed asymptotes are shifted (figs 1 and 2).

The data for saturating concentrations of DPG or IHP show a shift for the lower asymptote towards higher oxygen levels, indicating the need for the T′ form of lower affinity. The value of Y at which the relative minimum in n occurs depends on the fraction of Hb with effector bound, whereas a perturbed T_2 state model produces a minimum only at 50% ligand saturation. Overall, the data for various concentrations of DPG would favor an extended model [8], rather than the perturbed T_2 state.

The data for oxygen binding to Hb + L345 are quite different. Over a range of a factor of 8 in effector concentration, the relative minimum in n remains near 50% ligand saturation (fig. 7). In this case, a variation of the T_2 population provides an explanation for the double maximum. This would indicate that the probability of the T_2 population is increased by the effector to a greater extent than those of the other substates. A similar effect was observed for the binding of a fluorescent analog of DPG [15], where there was an enhancement of the signal from doubly liganded forms [16].

4.3. Existence of all ten tetramer states

The two-state model predicts an equal spacing of the energy levels for the substates. This implies a shift in equilibrium by the same factor $(1/c)$ for each ligand bound, yet this shift is difficult to test for all ligation levels. Because the equilibrium shifts by a factor of 100 for each ligand, it is difficult to measure the equilibrium for more than one ligation value. If the forms with two ligands (T_2 and R_2) exist in roughly equal amounts, then the singly and triply liganded tetramers will favor one allosteric form by nearly a factor of 100. Since the population of the partially liganded forms is low, an accurate determination of multiple equilibria is not possible from the OEC alone.

4.4. What evidence is there that all substates exist?

Using the perturbed two-state model for typical solution conditions (pH 7, $I = 0.1$ M without strong effectors), the probability for the substate R_0 and T_4 can be decreased without affecting the simulated curve. This is because they are very sparsely populated at all oxygen levels and contribute little to the OEC. From oxygen equilibrium data alone, there is no evidence to show that they are present. Similarly, the substates R_1 and T_3 are difficult to detect. There is therefore a problem in determining whether a shift in energy of a substate should propagate to the next ligation level. For example, in a model incorporating a heme-heme interaction for certain doubly liganded forms, this interaction may be assumed to persist upon addition of the third and fourth ligand [17]. If the T_2 substate is stabilized, it seems that the interaction should also be present for the substates T_3 and T_4. However, the populations of T_3 and T_4 are usually too low to determine whether they incur the same change in probability as for the substate T_2.

5. Conclusions

The two-state model can simulate most of the OEC for Hb and provides a practical and physical model for describing the equilibrium data. There are clearly equilibrium curves which are not well simulated by the two-state model. Additional allosteric states or internal interactions can be used which improve the fit, usually in proportion to the number of parameters used. We have described an

alternative scheme for fine tuning a given equilibrium curve. In the absence of hard evidence for new allosteric forms, this perturbed model maintains the same two-state framework and considers the effect of slight imperfections in the energy levels of the original substates. This method of simulation can help one to determine at which substate the Hb in question deviates from the two-state model. Detailed physical models can then be constructed to describe the additional interaction energy.

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